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During the first year of this project, we have organized and trained the required personnel for this project. We have begun work on all aims except Aim 5. Specifically, we have set up a quantitative cell transplant assay; worked out most problems with the Big Blue® assay; designed the radiation fields and procedures for the carcinogenesis experiments and begun collecting mammary DNA from age specific rats for the differential gene expression assay.

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## INTRODUCTION

Our ability to prevent breast cancer by rationally designed intervention requires a better understanding of the etiology of this disease. While the incidence of breast cancer continues to rise, our understanding of the causes underlying both this disease and its increasing incidence are poorly understood. Classical and molecular epidemiology has significantly improved our understanding of the etiology of several cancers such as lung, gastric, head and neck, and bladder. Most epidemiological studies of breast cancer have failed to identify major factors underlying the initiation of breast cancer even though several modifying factors that either promote or inhibit breast cancer development have been identified. A possible explanation for the inability to identify breast cancer initiating agents is suggested by the intensive epidemiological studies of breast cancer etiology in the survivors of the Hiroshima and Nagasaki A-bomb radiation exposures. These studies showed that radiation, even at low doses, causes breast cancer (1). The greatest risk of developing breast cancer was among those exposed when less than ten years of age. The risk of those exposed when 10 to 19 years old was slightly but significantly lower. Risk decreased rapidly with age at exposure thereafter; women over 40 were only minimally susceptible to radiation carcinogenesis (1).

These findings suggest that epidemiological studies that seek environmental and lifestyle factors underlying breast cancer initiation may lose much of their power by restricting their observations to cohorts of middle-aged women (the group that is usually included in most published epidemiological studies). While breast cancer frequently occurs in this middle-aged population, this group of women may have only a minimal sensitivity to the various environmental agents under study, and breast cancer initiation is likely to have occurred decades earlier under a very different environment/lifestyle than is current for this older cohort of women. In addition to studies of radiation carcinogenesis of A-bomb survivors, similar age versus risk trends have been reported in both medically irradiated patients (2), and in several epidemiological studies of cigarette smokers (3). For example, Brinton et al. report an increased relative risk to breast cancer in women who began smoking before age 17 (3). This is consistent with our in vitro studies with primary cultures of human breast epithelial cells in which we demonstrated the genotoxicity of cigarette smoke condensates (4).

Since the first reports of the strong dependence of age at exposure to radiation on breast cancer risk, its importance has been widely recognized; however, its underlying mechanism remains obscure. Before the current epidemiological data set became available, it was only clear that younger females were more susceptible to breast cancer initiation than older women. Accordingly, it was speculated that susceptibility was related to breast development and specifically to the increased rate of parenchymal cell division during breast maturation in the teenage girl. This hypothesis is supported by the data of Russo et al. (5) that shows that susceptibility to DMBA-induced mammary cancer in rats occurs at a period of high breast cell division as the rat becomes sexually mature. In contrast, the most recent epidemiological analysis published last year includes cancers detected in A-bomb survivors through 1985 and shows that the age at exposure for maximum risk of breast cancer initiation by radiation is before puberty and the accompanying menarchal increase in breast cell mitosis (1). Thus the full data set suggests that some aspect of the biology of the immature human breast results in maximal sensitivity to breast cancer initiation. It should also be kept in mind that while the immature breast is most susceptible to breast cancer initiation, the maturing breast (teens to early 20's) is

more susceptible to initiation than the breast of middle-aged, pre-menopausal women. This intermediate sensitivity may be rooted in the increased mitotic rate of the maturing breast parenchyma. Thus at least two physiologic processes may underlie susceptibility to breast cancer initiation: that associated with the immature breast and that associated with the developing breast. The literature has provided little to explain the former observation mechanistically; however, we have recently generated a novel hypothesis that may explain it.

We have shown that the breast stem-cells of sexually immature rats are highly sensitive to the cytotoxic effects of ionizing radiation (6). This sensitivity decreases and then disappears as the rats begin to mature (6). These data have led to our central hypothesis that the inability of the mammary parenchyma of the immature rat to recover from radiogenic cellular damage may underlie an increased susceptibility to radiation-induced genetic lesions that lead to the genesis of breast cancer. It is further hypothesized that this sensitivity to radiation can be extended to other environmental agents. Defining and characterizing the role of the high radiosensitivity of the immature mammary gland in a rat model will provide key data for future studies to assess interspecies extrapolation to women.

Sensitivity to Radiation Cytotoxicity of Mammary Cells as a Function of Age

In order to investigate the effect of age on radiation sensitivity, we irradiated rats at various ages ranging from 1 through 12 weeks of age and assayed their mammary clonogenic (stem) cells' survival. When immature rats were irradiated with 5 Gy (500 rad) when 1, 2, 3 or 4 weeks of age, 10% of the mammary cells survived. In contrast, a 3-fold increase in survival of irradiated mammary cells was observed when mature rats at 8 or 12 weeks of age were irradiated. When complete radiation dose versus cell survival curves were generated, not only did we confirm the quantitative difference in survival of mammary cells from immature and mature rats at all doses tested, but we also found a qualitative difference in the shapes of the survival curves between prepubertal and post-pubertal rats. Mammary cell survival curves from irradiated 2- and 4- week-old rats were straight on a semilog plot, indicating a purely exponential cell killing. In contrast, survival curves of mammary cells from 8-week-old rats had an initial shoulder region followed by a terminal exponential portion more typical of mammalian cells. The most common explanation for an initial convex shoulder on a survival curve suggests that it reflects an ability of the cells to recover from low to moderate doses of radiation. It may be further speculated that since the major damage leading to cell death following radiation is damage to DNA, this recovery may consist of DNA damage repair. In addition to cell death, nonrepaired or misrepaired DNA damage can lead directly to mutation and thus indirectly to neoplastic transformation. It is thus speculated that the extremely high sensitivity of the immature human breast to radiation carcinogenesis may result from an increased accumulation of damaged DNA due to a diminished ability to correctly repair DNA damage.

A second period of increased sensitivity may also occur during sexual maturation and gland development. This period of intermediate sensitivity in women may be related to an increased mitotic rate during gland growth at sexual maturation (5).

The relative importance of the two periods of increased sensitivity to breast cancer susceptibility may be species dependent. In order to have the greatest chance of correlating the human and rat results, this proposal will focus on the period of highest sensitivity in the human: the immature gland. We will extend our initial observation of increased radiation sensitivity on cell killing during a homologous period in the rat and test the endpoints of DNA damage,

mutagenesis and carcinogenesis. We will also investigate possible molecular mechanisms underlying the increased radiation sensitivity of the immature mammary parenchyma.

Mechanisms Underlying Radiation Sensitivity of the Immature Mammary Gland

Knowledge of the cellular and molecular biology underlying radiation damage has been expanding rapidly over the last several years. Our group has found that epidermal growth factor (EGF) can modulate radiation sensitivity. We have shown that in two human primary epithelial cell culture systems, breast (7) and prostate (8), the removal of epidermal growth factor from a defined growth medium before and during radiation increases the radiation sensitivity of both cell types. This effect has been shown to be independent of proliferation status (7,8). Others have shown that the removal of EGF inhibits the repair of radiation DNA strand breakage (9).

The in situ mammary gland produces both EGF and transforming growth factor α (TGFα) which signal through the same receptor. However, it is suggested on the basis of cellular and glandular distribution that only TGFα acts within the parenchyma of the gland via an autocrine/paracrine mechanism, whereas EGF is often apically secreted and not locally active (10). In elegant studies, TGFα mRNA was not found to be present in the immature mouse mammary gland; however, it is readily detectable in the maturing gland and persists in the adult virgin gland (10).

We hypothesize that the lack of TGFα in the immature gland leads to the observed increased radiation sensitivity that could extend to mutagenic and oncogenic sensitivity in this immature tissue. We plan to test this hypothesis directly as well as to further explore alternative molecular mechanisms underlying this age-dependent increased radiation sensitivity in the mammary gland.

Establishing the cellular and molecular mechanism underlying the increased sensitivity of the immature breast to carcinogenic environmental exposures will possibly lead to better designs for breast cancer epidemiological studies and to new prevention strategies. For example, if we show that it is likely that the radiation sensitivity of the immature breast extends to chemical xenobiotics then it would be suggested that epidemiological studies that seek agents that initiate breast cancer focus on young girls. Secondly, for example, if we demonstrated that this increased sensitivity of the immature breast is due to a low level of mammary gland associated TGFα then this would suggest new pharmacologic breast cancer prevention approaches using either TGFα or preferably non-peptide small molecules with TGFα activity.

PURPOSE

The overall goal of this proposal is to explore the hypothesis that the diminished ability of mammary cells from immature rats to recover from cytotoxic radiation damage may extend to an increased susceptibility to mammary carcinogenesis. If so, such a mechanism may also underlie the observation that the immature breast of prepubertal human females is the developmental stage most highly susceptible to breast cancer initiation.

It is further hypothesized that this low capacity of cells in prepubertal glands to recover from radiation damage is the result of a maturation stage-related lack of TGFα production. TGFα

apocrine/paracrine interaction and its resultant intracellular signaling may be needed to provide the required gene expression for cellular recovery from radiation damage.

SPECIFIC AIMS

In order to achieve our overall goals, we will address the following specific questions using a rat model:

1. Does the sensitivity to cell killing by ionizing radiation in immature glands extend to various classes of xenobiotic chemical carcinogens including those acting via bulky adducts (DMBA) and alkylating small adducts (NMU)?
2. Does the irradiation of cells from immature mammary glands (in contrast to mature glands) result in a higher sensitivity to the induction of specific locus mutations? Is the spectrum of mutations different in cells from immature and mature glands?
3. Is the immature gland more sensitive to the scopal carcinogenic effect of radiation?
4. Does irradiation of the immature gland (in contrast to the mature gland) result in a) more extensive DNA damage, b) more poorly repaired damage, or c) a greater induction of apoptotic cell death?
5. Is the lack of TGFα production by cells of the immature mammary gland related to the increased sensitivity of radiation-induced cell killing?

How is the spectrum of gene expression in the immature and mature mammary glands different with regard to genes which could directly or indirectly confer altered cellular recovery capacity following cytotoxic and genotoxic damage?

During the initial year of this project, most efforts were devoted to establishing and modifying the required methodology for each aim of the project. In addition, certain personnel were trained in these needed technologies.

BODY

**Aim 1: Does the sensitivity to cell killing by ionizing radiation in immature glands extend to various classes of xenobiotic chemical carcinogens including those acting via bulky adducts (DMBA) and alkylating small adducts (NMU)?**

During the first year, we reintroduced the needed transplant technology into the laboratory and validated it in the F344 rat. We are in the process of starting to run the proposed assays with the chemical carcinogens 7,12-dimethyl(a)benzanthracene (DMBA) and nitrosomethylurea (NMU). We anticipate completing this aim in year 2.

**Aim 2: Does the irradiation of cells from immature mammary glands (in contrast to mature glands) result in a higher sensitivity to the induction of specific locus mutations? Is the spectrum of mutations different in cells from immature and mature glands?**

An initial experiment was performed using two eight-week old Stratagene Big Blue® transgenic rats, one exposed to 2.75 Gy and one control, with a two week expression period. The mammary glands were isolated (left and right separately) and organoids were prepared; livers

and spleens were also harvested. Stratagene's DNA isolation protocol, which is designed for larger amounts of tissue, was followed for the DNA isolation. There was too little tissue to yield DNA; no results were obtained.

The next experiment, using Fischer 344 rats, (the background rat strain for the Stratagene Big Blue® transgenic rats) was designed to determine the number of rats necessary to yield sufficient tissue to isolate DNA for the assay. The rats were treated using a modified procedure based upon the reduced amount of available tissue. It was determined that with a modified DNA isolation procedure, 1/2 rat (left or right glands) would be sufficient for a satisfactory DNA yield.

Frozen liver and spleen from the initial experiment were processed according to the Stratagene protocol. There were no mutant blue plaques visible, but the four shades of color controls did not show blue plaques, indicating that the system was not working properly. Organoids and DNA were prepared from frozen glands, and the DNA from the liver and spleen samples was repackaged. No plaques were formed, indicating again that the assay was not working properly. Suggestions from Brenda Rogers at Stratagene were to decrease the pH of the bottom agar. She also recommended examining the DNA for phenol, which can decrease packaging efficiency and using a different lot of phenol:chloroform because doing so had alleviated difficulties they were having at Stratagene.

The next experiment was designed to test these suggestions. Two batches of bottom agar were prepared: pH 7.0 (normally recommended) and pH 6.5 (the lower pH recommended by Brenda Rogers). The color controls were plated, as were DNA samples from the previous experiment, DNA samples prepared with new reagents, and DNA samples isolated at the instructional workshop at Stratagene (samples known to give blue mutant plaques). No blue plaques were visible in any of the tissue samples; only the darkest color control showed blue plaques and they were far lighter blue than they should have been. The pH was irrelevant. Information was obtained from the Stratagene tech-line: 1) Placing the plates at 4°C for an hour prior to reading them should enhance blue color (it didn't in later trials) 2) Lot - to - lot variations in the casein hydrolysate could be causing the problems.

Two more rats were treated as in the first experiment. Right and left glands, livers, spleens, kidneys, lungs, and brains were harvested and frozen for later use. After following the modified DNA isolation procedure for organoids and the Stratagene protocols for the remainder of the procedure, no plaques were found on the plates. (It was later learned that DNA cannot be successfully isolated from frozen mammary gland. This explains the absence of plaques in the samples isolated from mammary glands.) Mutation frequencies were determined for the unirradiated liver ($4.8 \times 10^{-5}$) and irradiated spleen ($3.8 \times 10^{-5}$); others could not be determined because there were no blue plaques. No color controls were run with this experiment.

After further conversations with the Stratagene technicians confirming that the casein hydrolysate was preventing the visualization of blue mutant plaques and learning that Stratagene did not have a lot that allowed such visualization in the controls, we next began a series of experiments designed to find a source of casein hydrolysate that would allow the visualization of blue mutant plaques. The four color controls were plated without satisfactory blue color production on media prepared using commercially-made media or casein hydrolysate from various sources: Sigma casein hydrolysate, Gibco NZY medium, BBL NZY medium, ICN casein hydrolysate, and Fluka casein hydrolysate. Stratagene eventually marketed its own media preparation, which did yield acceptable blue color controls. However, the experiments with the old Stratagene Big Blue® transgenic system were begun May 29, 1996; the Stratagene satisfactory

media was not available until November 22, 1996. Effectively, it was impossible to perform an experiment with this system until then.

In the next experiment, two eight week-old old Stratagene Big Blue® transgenic rats were utilized. One was exposed to 3 Gy with a two week expression period; another served as a control. Organoids were prepared from left and right glands and frozen. Livers, spleens, kidneys, lungs, and brains were harvested and frozen. DNA was prepared using the Gentra DNA isolation protocol, which does not involve phenol:chloroform. The rest of the procedure was performed according to the Stratagene protocol. The color controls appeared as they should. Mutation frequencies were determined for the following samples: control liver, $5.2 \times 10^{-5}$; control spleen, $9.4 \times 10^{-5}$.

The final experiment performed compared the performance of the Gentra DNA isolation procedure to the standard phenol:chloroform procedure. DNA was prepared from frozen Big Blue® transgenic rat liver and spleen samples using the Gentra protocol, a modification of that protocol without vortexing (to reduce shearing of genomic DNA) and the traditional phenol:chloroform protocol. The DNA was then packaged according to Stratagene's protocol, and the number of plaques per microgram of DNA was determined. The modified Gentra protocol gave the best results and will be used in the ensuing experiments.

The next experiment is designed to determine the efficacy of the Big Blue® transgenic rat system for detecting mutations in the mammary gland. As yet, there are no reports in the literature of this system being used for the mammary gland, and previous attempts have not been conclusive. A small experiment will involve the dosing of Big Blue® transgenic rats with ethylnitrosourea, a known potent mammary mutagen, with an appropriate expression period. Using this experiment as a positive mammary control, we will proceed as planned over the next year.

**Aim 3: Is the immature gland more sensitive than the mature gland to the scopal carcinogenic effect of radiation?**

We have worked on the dosimetry for selectively irradiating mammary tissue in live rats. Following anesthesia by AALAC approved methods, animals will be placed in custom made holders. We have finished the holders for the eight week old rat and are in the process of modifying one which will fit the smaller three week old rat. We then plan to produce electrons of specific energies to be dictated by the anatomy of each sized rat. Irradiation will be performed using our departmental based linac-accelerator (Varian). Dosimetry will be accomplished by the PI with the help of Dr. Bhudatt Paliwal, who is the chief of physics for our radiation therapy clinical unit.

**Aim 4: Does irradiation of the immature gland (in contrast to the mature gland) result in a) more extensive DNA damage, b) more poorly repaired damage, or c) a greater induction of apoptotic cell death?**

During the first year, we established the comet assay in our lab. This involved designing hardware and writing customized software followed by setting up the biological assay.

Development of a Semi-automated Assay for Evaluating the Results of a Comet Assay

Comets are viewed using an Olympus epifluorescence microscope under green light excitation (510-590 nm) from a 100 W mercury source. A 40X objective is used to view individual comets, while a CCD camera attached to the microscope collects the images for digitization. The camera signal is passed to an IC-PCI image capture board and AM-CLR color acquisition module (Imaging Technology, Bedford, MA) with a 32-bit connection to the host CPU. Images captured in the IC-PCI's memory are rapidly transferred over the PCI bus and displayed on the host computer's screen, allowing the real-time digitization and viewing of comet images. Developing this hardware required a period of approximately two months.

The image acquisition and analysis software application was written by our group in the C++ programming language. It uses a few subroutines in the ITEX-IC and ITEX-IPL libraries (Imaging Technology, New Jersey) for processing and hardware configuration. The program allows the complete analysis of up to 300 comets/hour, with the output directly written into data spreadsheets. Each analysis session organizes the following information into the user-specified spreadsheet: 1) tail moment, 2) percent DNA in tail, 3) tail intensity, and 4) total comet intensity for each comet analyzed; plus, it generates histograms of binned values of tail moment and the percent of the DNA in the tail. The application was written to be controlled through a DOS window in a very simple and instructive manner, with a user needing only minimal understanding of the actual analysis methods. Users need only locate and focus images and identify the geometrical center of each comet head. The number of comets analyzed per hour is determined solely by how efficient a user is at this identification process.

A separate analysis session is required for each experimental sample, which come in the form of separate slides. A session begins by prompting the user for an output file name and also the dimensions of the comet region of interest (ROI). These dimensions, typically 300 x 200 pixels, are determined by the relative size of the cells being studied and can be varied accordingly. The program then initializes all the hardware and starts grabbing live images from the camera. Comet fields are viewed as 512 x 480 pixel images with 256 intensity levels. One by one, the user moves and focuses comet images inside the ROI with the microscope stage controls, making sure to avoid overlapping comets or comets with non-uniform background. Once focused, an image is snapped into host memory as a 2-D tiff file, and the user moves a vertical line in the image window to locate the center of the comet head. Using the pixel intensities of the image and the coordinates of the center of the comet head, the program instantaneously computes all the desired values and writes them to the specified spreadsheet. The image window then returns to grabbing live images, at which point the user scans forward to the next comet on the slide, and the process continues.

Each two-dimensional intensity plot is integrated perpendicular to the direction of electrophoresis to yield a one-dimensional intensity profile. Next, background is subtracted based on the average background intensity of each acquired image. After the background subtraction, the lagging edge of the comet head is mirrored about the center, then subtracted from the comet profile to give the tail profile. The comet and tail profile are each integrated to give the total comet intensity and tail intensity, respectively. The fraction of DNA in the tail is simply the ratio of tail intensity to total comet intensity. From the tail profile, the tail moment is calculated by integrating the product of the distance from the center and the intensity value at that distance. Completing this integration from the center of the comet head to the end of the tail and dividing the result by the total comet intensity yields the tail moment.

Optimization of the UW Comet Analysis System

The first comet assay performed was an alkaline assay because while the cell and slide preparation are the same between the two assays, the alkaline assay can be completed more rapidly than the neutral assay; it could be determined more quickly whether the process had been successfully performed. V79 cells were used because they had been used extensively in the comet assay by others, thus results could be compared and the success of the process verified.

In the first experiment, 85% confluent cells were used. They were trypsinized to remove them from the flask in which they had been growing. Media was added to stop the trypsinization, and the cells were centrifuged. The cell pellet was resuspended in 1 ml of calcium and magnesium-free phosphate-buffered saline (PBS). After dilution, the cells were counted using a hemocytometer. The cells were diluted to $2x10^4$ cells/ml. A cell-agarose solution was pipetted onto pre-coated fully frosted slides and allowed to gel. The slides were placed into alkaline lysis buffer and allowed to lyse in the dark for one hour before rinsing for one hour in three changes of alkaline rinse solution in the dark. The slides were electrophoresed for 25 minutes at 0.6 volts/centimeter. The slides were then rinsed in distilled water and stained with propidium iodide. They were rinsed with distilled water and stored in an air-tight container in the dark in the cold room. Difficulties were encountered with the analysis for a number of reasons: 1) the cell concentration needed to be increased to $1\text{-}2x10^4$ cells/slide and 2) the agarose needed to be at a lower temperature before the cell suspension was added. Once these difficulties were corrected, more extensive experiments could be conducted.

The next experiment was performed on cells that had been irradiated with 12 Gray from a cesium source and unirradiated control cells. The control cells were treated like the irradiated cells in all respects except the irradiation. The flask was positioned upright in the irradiator in order to fit in the accurate-dosing range. At all other times, the flasks were positioned such that the cells would be covered with media. After irradiation, the cells were treated as described above. Two slides were prepared from cells receiving each dose. The composite histograms are shown as Figure 1. There was a slight shift in tail moment between the control and irradiated cells, but it was not as dramatic as expected.

The next experiment was designed to generate a radiation dose-response curve for the alkaline and neutral assays. Five flasks of cells were prepared, receiving 0, 4, 8, 12, or 16 Gray of radiation from a cesium source as described above. The neutral and alkaline assays were performed simultaneously using the same cells. One slide was prepared for each dose for each assay. The neutral assay differs from the alkaline assay in the following ways: 1) The lysis step in the neutral assay is performed in a neutral protein removal buffer for four hours at 50°C. 2) The rinse step prior to electrophoresis is performed in TBE buffer overnight in two changes of buffer. The histograms are shown as Figure 2. The analysis was difficult because there were still too many cells on each slide.

Two more trials of the dose-response experiment were performed in order to attempt to replicate the results. The only difference was that a lower concentration cell suspension was used to prepare the slides in order to simplify analysis. The results of one of the experiments are shown as Figure 3. Notice how the histograms are much tighter. This is a more desirable result than the widely-spread histograms shown in Figure 2. Figure 4 shows the results of the second experiment. Again there are tight histograms except for the 12 and 16 Gy alkaline assays. Figure 5 shows the dose-response curves of the three experiments. Such curves should have a positive

slope -- much more positive than these are. After this experiment, it was discovered that the problem was in the method of irradiation. In order for the cells to be damaged by the irradiation, they need to be more oxic. Irradiation conditions are being modified to allow for better oxygenation.

**Aim 5: Is the lack of TGFα production by cells of the immature mammary gland related to the increased sensitivity of radiation induced cell killing?**

This aim has not yet been worked on.

**Aim 6: How is the spectrum of gene expression in the immature and mature mammary glands different with regard to genes which could directly or indirectly confer altered DNA repair capacity?**

Rat mammary glands of each side of the body were designated as A through F from head to tail. Mammary tissue of glands D, E and F are easier to separate from the surrounding tissue than glands A, B and C, and therefore were chosen for our study. Four 23-day old Fischer 344 rats were sacrificed for epithelial cell staining and total RNA isolation. The staining of right-side glands showed that E gland contains more epithelial cells than D or F glands. The left E glands from the same four rats were homogenized in RNazol B reagent (Tel-Test) to isolate the total RNA. The yield of total RNA ranged from 26.8 μg to 52.6 μg per gland E. By extrapolation, the yield from E glands per rat would be 53.6 μg to 105.2 μg. We will need approximately 2 mg total RNA from 3-week old rats for mRNA isolation and other applications. Based on the above calculation, we will need 30 three-week old rats for our initial study. We also need 10 eight-week old rats.

To identify genes that are differentially expressed between 3-week old and 8-week old rats, we will initially apply a suppression subtractive hybridization (SSH) method. Total RNA is isolated from mammary glands using RNeasy kits (Qiagen) and subjected to mRNA purification with Oligotex mRNA kits (Qiagen). Tester and driver double-stranded (ds) cDNA are prepared from the two mRNA samples under comparison. Tester and driver cDNAs are separately digested with *Rsa*I to obtained shorter, blunt-ended molecules. Two tester populations are created with different adapters, but driver cDNA has no adapters. Each tester-adapter population is mixed separately with 30-fold of driver cDNA in the first hybridization. Hybridization kinetics lead to equalization and enrichment of differentially expressed sequences. Then the two tester-driver populations are mixed for a second hybridization to generate templates of differentially expressed sequences for two rounds of PCR amplification. The final PCR products are cloned into plasmids to generate a library for further screening. This assay will then be followed up using our subtractive display assay.

## CONCLUSIONS

During the first year, most efforts were devoted to setting up experimental assay systems. This will provide a strong base for experimentation in year 2. Thus far, no definitive data have been generated from which to draw specific conclusions for the hypotheses under study.

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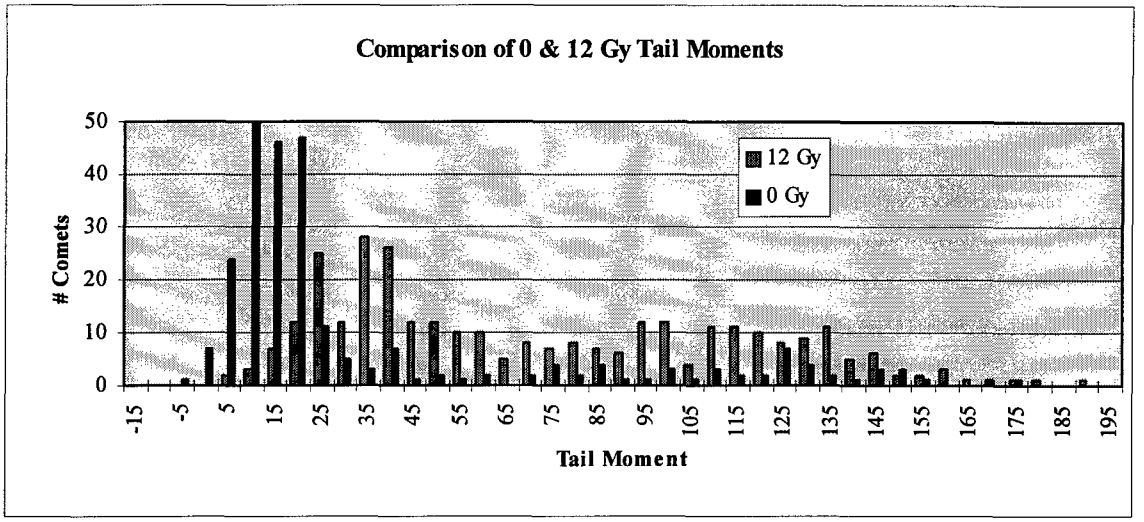


Figure 1. I324 Histograms

The 0 Gy histogram is tight, which is desirable. The 12 Gy histogram is more diffuse and slightly shifted to the right due to higher tail moments as a result of the damage caused by the irradiation.

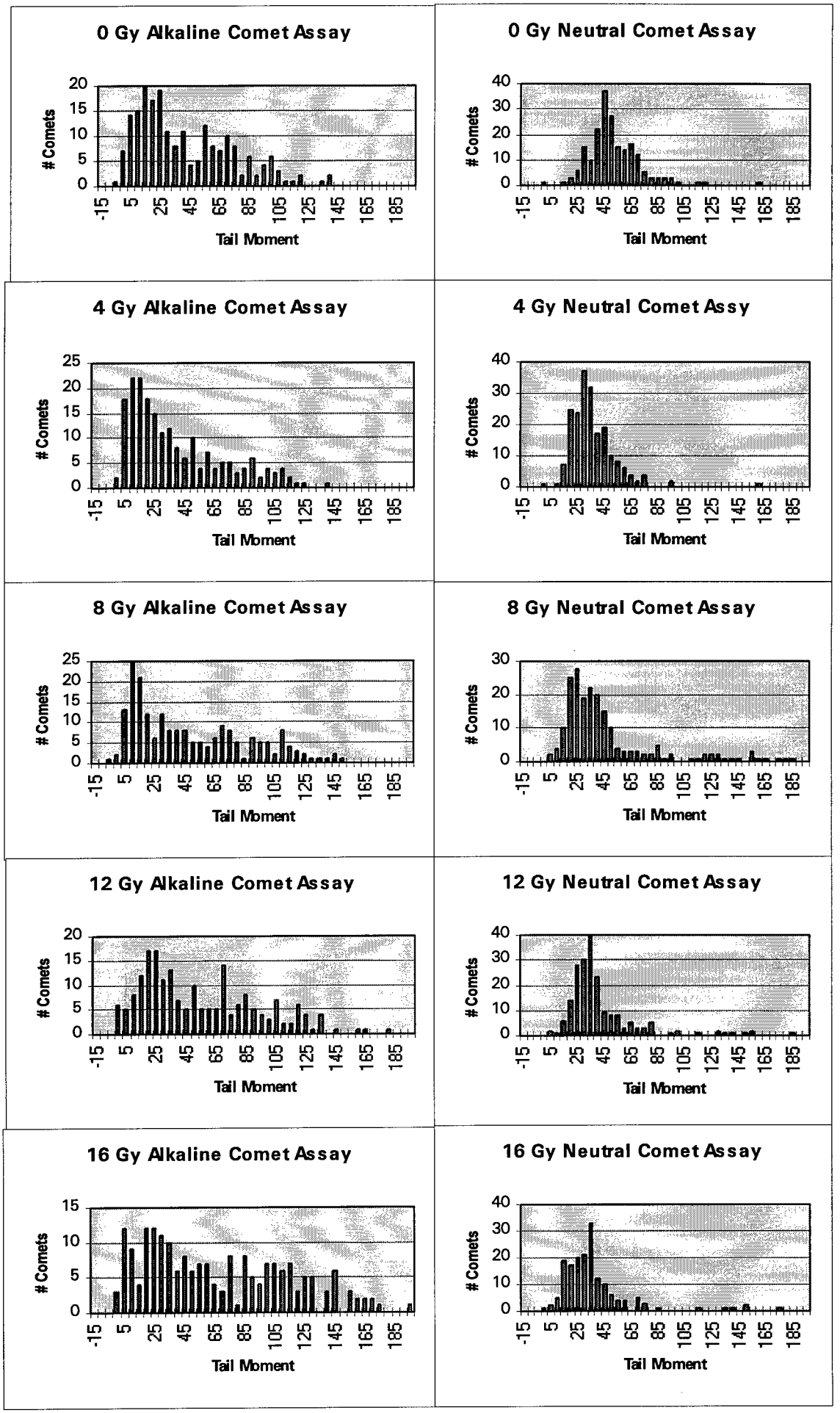


Figure 2. I330 Histograms

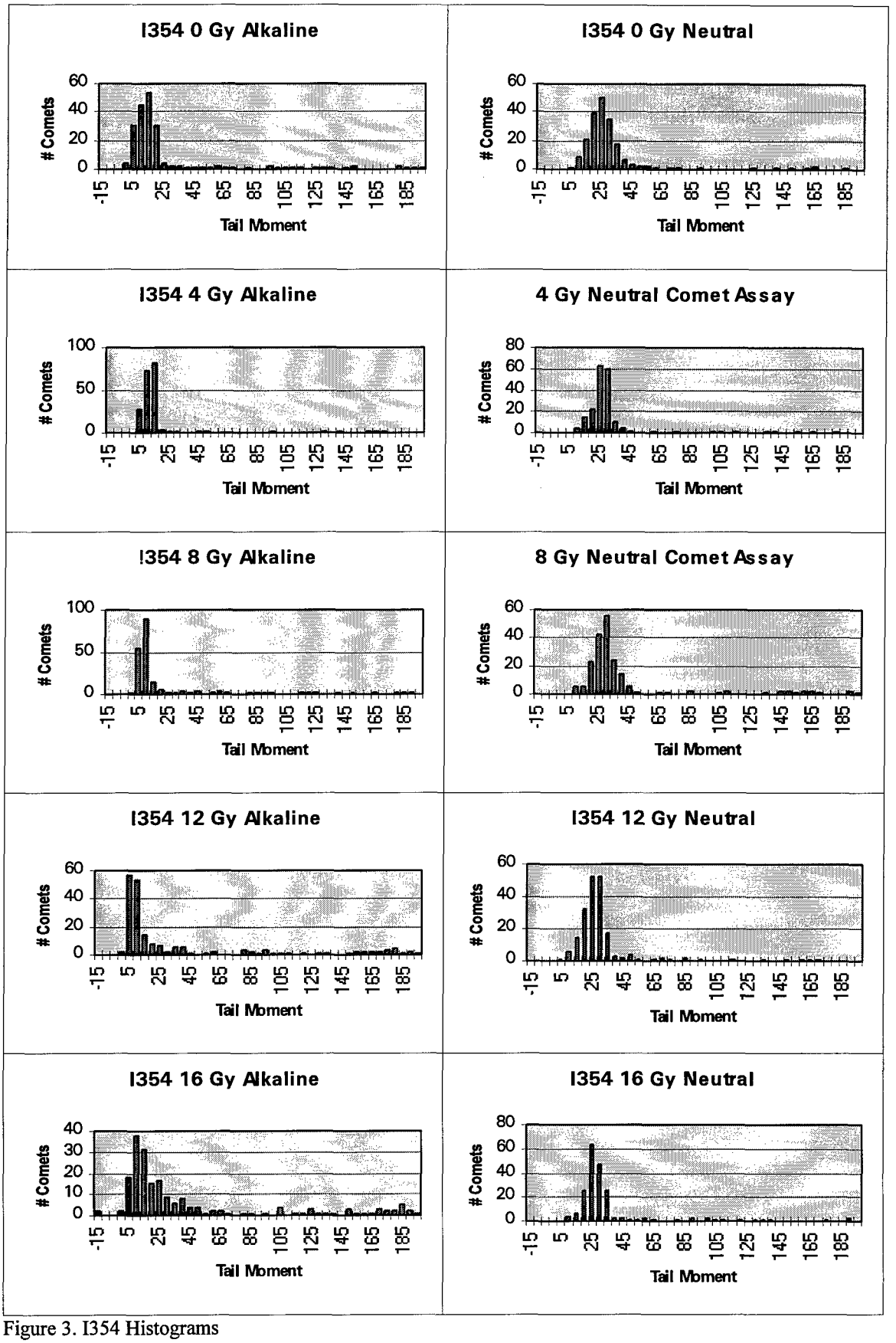

Figure 3. I354 Histograms

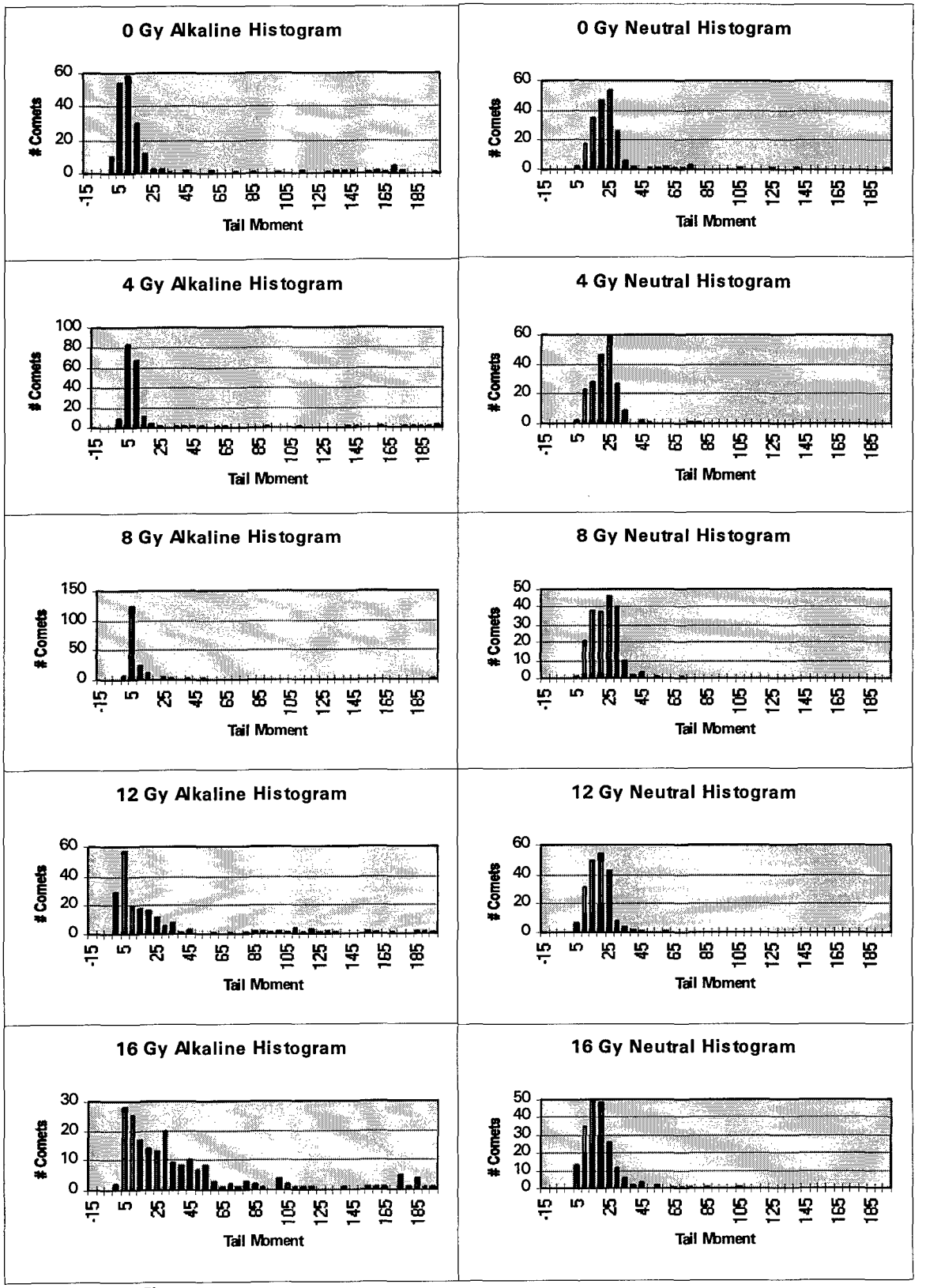

Figure 4. I379 Histograms

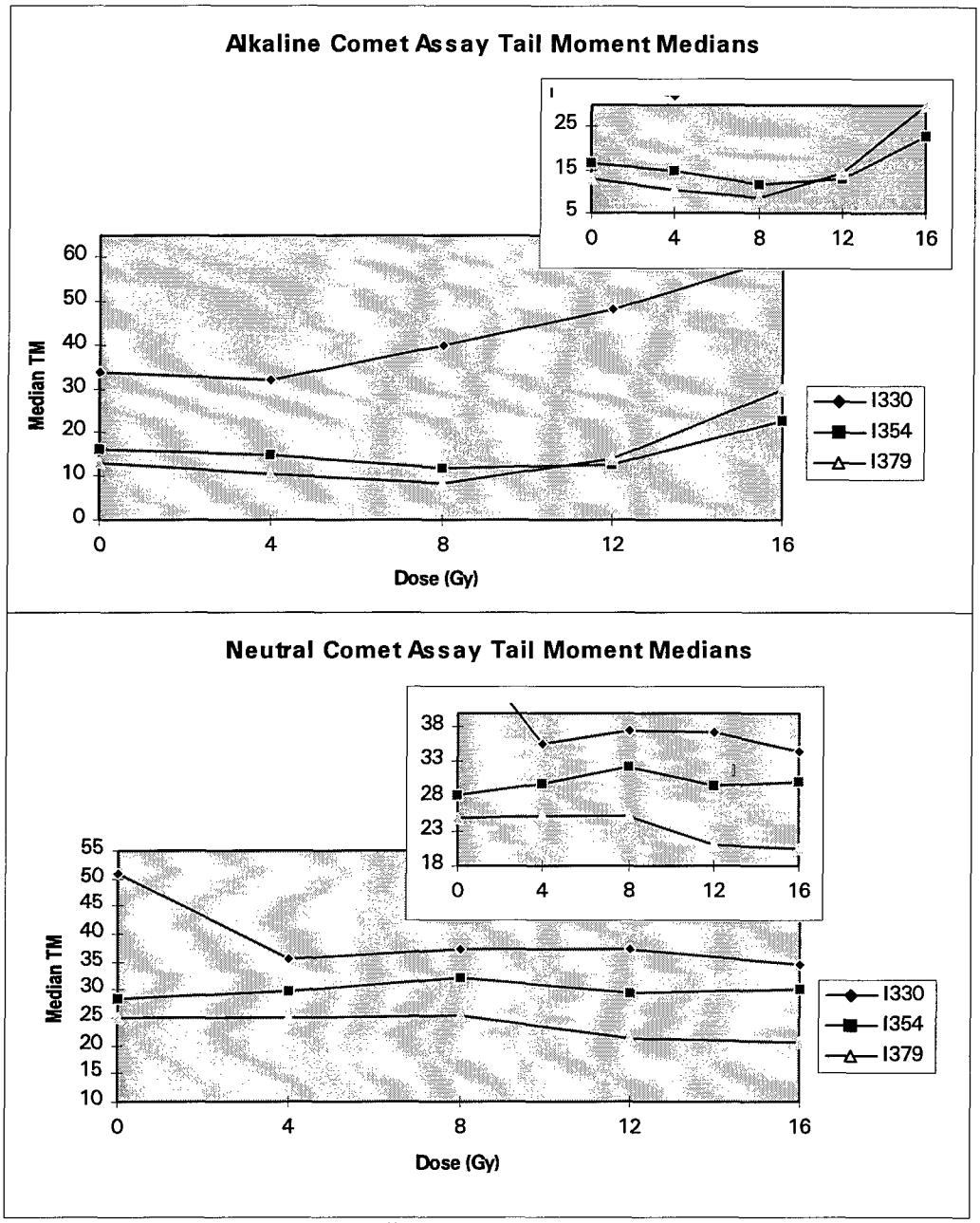


Figure 5. Median Tail Moments of All Dose-Response Data
The insets are expanded versions of the full graphs. Ideally, there would be a much steeper positive slope in these graphs. This failure can be corrected by irradiating the cells with media on them. The exact values of the median tail moments are not expected to coincide, but the trends should be the same.